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The Effects of Lead Acetate on Host Susceptibility to Trypanosoma Cruzi

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THE EFFECTS OF LEAD ACETATE ON HOST SUSCEPTIBILITY TO *TRYPANOSOMA CRUZI*

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Trypanosoma cruzi, a protozoan parasite, causes American trypanosomiasis or Chagas' disease. The infective stage of this parasite resides in the hindgut of the reduviid bug (family Reduviidae), which is both host and vector, and is transmitted to the mammalian host through fecal material released during a blood meal. Chagas' disease, an acute to chronic infection resulting in fever, malaise, and heart and liver enlargement, is becoming a concern in the United States due to the large increase in Latin American immigrants, and the lack of safe and effective therapeutic treatment and vaccination. Another growing concern in the United States involves environmental toxicants and their ability to act as immunosuppressants. Lead is of particular interest because it is widespread in places such as soil, food, water, batteries, paints, and plastics. Studies have shown lead exposure during bacterial and viral infection causes decreases in antibody production, T cell recognition, and macrophage activity--all of which result in decreased host resistance to infection. The present study was conducted to determine the immunosuppressive effects of lead acetate in C57Bl/6 mice challenged with the Brazil strain of *T. cruzi*. Mice were treated with

lead acetate concentrations ranging from 0 to 1000 p.p.m. in their drinking water for three weeks prior to infection. During the fourth week of the experiment, mice were injected with $1x10^4$ blood-form trypomastigotes (BFTs) of T. cruzi and lead treatment was continued. Food and water consumption was measured to monitor feeding patterns and lead exposure during infection. Parasitemias were performed at fourteen days post infection and continued at 3 and 4 day intervals throughout the study. Parasite-specific antibody production was determined by an indirect enzyme-linked immunosorbent assay (ELISA) using serum samples collected during high parasite counts. Average daily food consumption showed no statistical differences between lead-treatment groups of mice per replicate experiment. Average daily water consumption and mean weight gain showed no statistical differences among groups in replicates II and III; however, in replicate I significant differences were observed in both variables between mice receiving 100 and 1000 p.p.m. lead acetate concentrations. The highest mean peak parasitemias were observed in mice receiving 100 and 1000 p.p.m. lead acetate, though high lead acetate concentrations did not appear to suppress T. cruzi-specific antibody production. Although low mortality was observed in lead-treated mice, the results of this study suggest that lead exposure does enhance the susceptibility of mice to infection with T. cruzi, resulting in high parasitemias.

INTRODUCTION

Toxicology

Toxicology has been defined by Newcombe (1992) as "the science dealing with chemicals that cause disease." For a chemical to be classified as a toxicant, it must enter the host organism through one of several exposure routes: dermal; oral; inhalational; or intravenous. Exposure time of toxicants may vary from acute (four-six hours) to chronic (three months to two years). There are many ways to classify a toxic substance including primary target organ, use of substance, exposure time, exposure route, physical state of substance, chemical qualities of substance, and biochemical processes used to eliminate the substance. Pesticides, radiation, animal toxins, and heavy metals are the toxicants which have been most studied to date (Houston, W.E. 1994, pers. comm.). Heavy metals have become a particular concern because many are found naturally. The effects of metals depend on several factors including concentration, duration of exposure, species and sex of the host, and route of exposure.

Immune Response Mechanisms

The immune system is complex and involves a variety of different cell types which interact with foreign substances in a host organism. The defense mechanisms

used by the host include innate immunity and acquired immunity. The innate immune response involves the general defense mechanisms of a host including anatomic, physiologic, endocytic and phagocytic, and inflammatory (Kuby, 1994). The primary characteristics of acquired immunity are specificity, diversity, self/nonself recognition, and immunological memory (Kuby, 1994). The host is able to mount a heightened and more rapid response to a repeated exposure to antigen due to immunological memory.

Cellular and humoral immunity are two mechanisms involved in acquired immunity. The cellular response consists of those reactions that operate through the specificity and direct activity of T lymphocytes, and manifest as either cell-mediated, delayed-type hypersensitivity (DTH), tumor and allograft rejection, or allogeneic disease (Kuby, 1994). Cell-mediated immunity involves the activity of $CD8^+$ T cytotoxic cells and occurs when the T cells respond to specific antigens. Delayed-type hypersensitivity, a type IV hypersensitive response mediated by T cells, is a response that occurs approximately two or three days after the T cell interacts with an antigen and involves the release of particular cytokines, such as, interleukins 2 and 3, tumor necrosis factor β, and interferon γ (Kuby, 1994). The DTH response is particularly important in defending the host against bacterial and parasitic infections.

Humoral immunity functions by producing antibodies which are circulated through the serum. There are five classes of immunoglobulin in humans, designated IgM, IgG, IgA, IgE, IgD. IgG and IgM classes of immunoglobulins are extremely useful in defending the host against extracellular bacteria and exotoxin-producing

bacteria due to their agglutinating properties and their ability to fix complement (Kuby, 1994).

Lymphocytes are important in both acquired and innate immunity. One type of lymphocyte, the T cell, arises from hematopoietic stem cells in the bone marrow and completes its maturation in the thymus gland (Kuby, 1994). T cells divide and become distinct as either helper, cytotoxic, or memory cells. The other type of lymphocyte, the B cell, arises from stem cells in the bone marrow and remains in the marrow until maturation. B cells produce antibodies and influence T cells by acting as antigen-presenting cells. $CD4^+$ T helper cells (class II major histocompatibility complex (MHC)-restricted) recognize antigen-class II MHC complex on an antigen-presenting cell. T cell receptor (TcR) recognition involves the formation of a tri-molecular complex (MHC + antigen + TcR) (Kuby, 1994). Once the complex is bound the T helper cell is activated to secrete a variety of cytokines which stimulates B lymphocytes to undergo clonal expansion. In this process, B cells divide repeatedly and differentiate into plasma cells (antibody secreting) and memory cells (expressing membrane-bound antibody) (Kuby, 1994). IgG and IgM class immunoglobulins participate in the enhancement of phagocytosis, neutralization of toxicity, and complement-mediated lysis (Caren, 1981; Kuby, 1994). $CD8^+$ cytotoxic T cells (class I MHC-restricted) destroy tumor cells and foreign cells upon contact. Memory T cells are responsible for the enhanced recognition response to a specific foreign substance. Most antigens require the participation of both T helper and B cells to stimulate an antibody response.

Macrophages play a major role in the immune response as antigen processing and antigen presenting cells. Macrophages are derived from monocytes, and both cell types are actively phagocytic. Macrophages and monocytes process and present the antigen in association with class II MHC molecules to $CD4^+$ T_h cells, secrete cytokines, and respond in antibody-dependent cell-mediated cytotoxicity (Kuby, 1994).

Immunotoxicology

The field of immunotoxicology combines knowledge from immunology and toxicology to determine the effects of toxicants on the immune system. As a discipline, immunotoxicology involves the study of opposing effects of occupational, inadvertent, or therapeutic exposure to drugs and environmental chemicals or biological products on the immune system of living organisms (Exon, 1990). The field of immunotoxicology was initiated in the 1970's when researchers began investigating the immunotoxic potential of two environmental chemicals, polychlorinated biphenyls (PBCs) and lead (Loblay and Neill, 1992). Through the 1970's and early 1980's, the number of studies increased and the international awareness of immunotoxicology grew (Exon, 1990).

Immunotoxicants can be xenobiotic (pharmaceuticals, cosmetics, food additives, agricultural chemicals, environmental pollutants, and natural toxins) or biological products (enzymes, hormones, vaccines, allergen extracts, blood products, monoclonal antibodies, interferons, interleukins, growth factors, and other cytokines) (Loblay and Neill, 1992). There are three modes of action by which xenobiotics may

induce immunosuppression: hormonal effects caused by xenobiotic-induced changes in normally secreted hormone levels; chemically-induced functional defects of cells in the immune response; and chemically induced depletion of responding cells (Faith *et al.*, 1980). Chemical induction, which results in a decreased number of responding cells correlates with defects in cellular activation or initiation of suppressor cells (Faith *et al.*, 1980). When the responding cells are affected, lymphocytes may die, blockage of lymphocyte maturation may occur, and there may be interference with lymphocyte activity (Faith *et al.*, 1980).

The immune system may respond to an immunotoxicant by suppression or enhancement of reactions directed against the chemical or one of its metabolites. Immunosuppression may result in increased susceptibility of the host to viral, bacterial, fungal pathogens, or cancer (Vos, 1977). Immunosuppressive compounds are typically drugs, alkylating agents, thiopurines, folic acid antagonists, pyrimidine nucleoside analogues, some antibiotics, industrial chemicals, pesticides, and heavy metals (Vos, 1977; Koller, 1979). An immunosuppressive substance may act upon rapidly dividing cells such as lymphocytes, hemopoietic, and reproductive cells (Vos, 1977). The effects on the immune system are not selective and can impact the immune system directly or indirectly.

One successful approach to determining if an environmental contaminant could affect the immune system, thus enhancing susceptibility to infection, is to inject the host with an LD_{50} dose of a bacterial or a viral pathogen during exposure to a toxicant (Koller, 1979). An LD_{50} dose is lethal to 50 % of the individuals given that dose. In

1971, Hemphill *et al.* studied the ability of lead to suppress host resistance to Salmonella typhimurium, hypothesizing that exposure to low concentrations of lead would cause mice to have decreased resistance to bacterial infection. Swiss-Webster mice were exposed to subclinical doses (either 100 μg or 200 μg) of lead nitrate for thirty days (Hemphill *et al.*, 1971). There were no overt clinical signs of toxicity observed during the observation period, despite indications of enhanced susceptibility to the bacteria. A Tenfold increase in mortality with an LD_{50} ($10^{-4.7}$) of S. typhimurium was observed in lead-treated mice relative to controls. Hemphill *et al.* (1971) concluded there was a negative impact of lead exposure on the immune system, causing decreased resistance of the host against S. typhimurium and resulting in high mortality. It is known that lead has the ability to bind to proteins *in vitro* and possibly *in vivo*. Hemphill *et al.* (1971) suggested the ability of lead to bind to various complement proteins, involved in antigen-antibody interaction, may interfere with the functional activity of the alternative and the classical complement pathways, thus decreasing the resistance of the host to S. typhimurium.

Prior to 1977, most of the studies involving lead focused on increased susceptibility to infectious gram-negative bacteria containing endotoxins (Vos, 1977). Seyle *et al.* (1966) studied the effects of lead on rats exposed to a bacterial endotoxin. A greater sensitizing effect was observed when lead and Escherichia coli endotoxin were given simultaneously (Seyle *et al.*, 1966). In this case, susceptibility to endotoxin was shown to increase approximately 100,000 times; when rats were treated with 100 μg of E. coli endotoxin, the death rate and histopathology was the same as

when the rats were exposed to 1 ng of E. coli and 5 mg/100 g body weight of lead (a well tolerated dose).

The effects of methylmercury, tetraethyl lead, and sodium arsenite on the humoral immune response of Swiss cross male mice also have been examined (Blakley *et al.*, 1980). This particular experiment lasted for three weeks, with groups of mice receiving concentrations of between 0.5 and 10.0 p.p.m. of methylmercury, tetraethyl lead, or sodium arsenite in their drinking water. The mice in this study did not show signs of toxicity to either methylmercury, tetraethyl lead, or sodium arsenite; however, maximum immunosuppression of antibody-producing cells and antibody production was found in all metal concentrations of 0.5 and 1.0 p.p.m. and somewhat less immunosuppression with 2.0 p.p.m. A dose-related effect on tissue-metal concentrations was observed at all metal concentrations when the kidney and the liver were analyzed (Blakley *et al.*, 1980). High lead concentration exposure resulted in high tissue-lead concentration. The authors of the study suggested that the mechanism of suppression may have involved impairment of macrophage function or depression of particular lymphocyte subpopulations (Blakley *et al.*, 1980).

Koller *et al.* (1979) conducted a study to determine if lymphocytes obtained from CBA mice treated with lead or cadmium would have an altered response to mitogen stimulation. Twenty-five mice (group 1) were given 13, 130, or 1300 p.p.m. lead acetate, and twenty-five mice (group 2) received 3, 30, or 300 p.p.m. cadmium chloride in their drinking water for ten weeks. Selected animals in each group were injected with Mycobacterium bovis (BCG). Neither lead nor cadmium had an

observable effect on T lymphocyte proliferation induced by exposure to the mitogen concanavalin A (Con A). Lead treatment did result in depression of the proliferative response of lymphocytes to the mitogens lipopolysaccharide (LPS) and purified protein derivative (PPD), while cadmium actually stimulated blastogenesis in response to these mitogens (Koller *et al.*, 1979).

Faith *et al.* (1979) investigated chronic, low concentration lead exposure on Sprague Dawley rats. Newborn female rats were given 25 and 50 p.p.m. lead acetate in their drinking water for seven weeks. The rats were mated with untreated males and continued on the lead-treatment through gestation and lactation. Male and female offspring were maintained on the same regimen as the mother, and between the ages of 35 and 45 days, were studied for immunological effects. Second generation rats showed no differences in growth rate and no clinical signs of toxicity were observed. Both male and female offspring had significantly lowered thymic weights at birth in each treatment condition relative to controls. Researchers found lead treatment depressed the ability of the lymphocytes to respond to mitogen stimulation with phytohaemagglutin-p (PHA) or Con A and delayed-type hypersensitivity to PPD challenge. The decrease in thymic cell proliferation in response to Con A was not significantly different among lead-treatment groups. T lymphocyte proliferation in response to PHA or Con A demonstrated a statistically significant decrease in both lead-treatment groups relative to controls. These results suggest that long-term exposure to lead may lead to immunosuppression of cell-mediated and humoral immune function (Faith *et al.*, 1979).

Koller (1973) analyzed the effects of lead, cadmium, and mercury on the humoral antibody response in rabbits exposed to a viral pathogen. New Zealand White male rabbits were exposed to 2500 p.p.m. lead acetate, 300 p.p.m. cadmium chloride, or 10 p.p.m. mercuric chloride for 70 days. No clinical signs of toxicity were observed. The animals were challenged with pseudorabies virus (Herpesviridae family) on day 71, with a second dose day 78, and a third dose on day 85. Serum samples were prepared on the same days for use in ELISA assays to determine antibody production. The lead-treated rabbits showed an approximate tenfold decrease in antibody production. Mean serum-antibody titers were significantly decreased in all treatment metal groups relative to controls. Mercury-treated rabbits showed the least decrease in antibody titers compared to other metal treatment groups. There also was a significant decrease in the packed cell volume and body weight gain of the lead-treated groups, though no such decreases were observed in cadmium- or mercury-treated rabbits (Koller, 1973).

Koller and Kovacic (1974) studied Swiss-Webster mice exposed to 13.75, 137.5, or 1375 p.p.m. lead acetate concentrations in their drinking water for 56 days. Mice were inoculated with sheep red blood cells (SRBC) and their antibody production was measured. The results showed a decrease in antibody production and numbers of B lymphocytes relative to controls (Koller and Kovacic, 1974). IgG antibody response was low, suggesting that memory cells were affected by lead acetate exposure. The longer the animals were exposed to lead, the greater the decrease of both IgM and IgG antibody production. Koller and Kovacic (1974) suggested lead acetate decreased the

number of antibody-producing cells, leading to a decrease in the level of circulating antibodies, and that the reduction in antibody production is responsible for the increased risk of bacterial or viral disease in animals with chronic exposure to lead.

Koller and Brauner (1977) also studied the effects of lead and cadmium exposure on B lymphocytes in CBA/J mice. Twenty-five CBA/J mice exposed to 13, 130, or 1300 p.p.m. lead acetate or 3, 30, or 300 p.p.m. cadmium chloride in their drinking water for 70 days. No toxic signs were observed. The number of erythrocyte-antibody-complement (EAC) rosettes were significantly lower in the mice treated with 130 or 1300 p.p.m. lead or 30 p.p.m. cadmium as compared to the control group. Exposure to lead and cadmium was associated with decreased formation of rosettes, thus accounting for the decrease in antibody response occurring during primary immunity (Koller and Brauner, 1977). Koller and Brauner (1977) suggested that, in this experiment, because the lymphocyte viability and percentage T and B cells were common between treatment groups, the complement receptor site on the surface of the B cell was altered by lead and cadmium exposure. A direct inhibition of B cells due to lead and cadmium exposure represents an obvious danger to the health of a host, as B cells produce antibodies (Koller and Brauner, 1977).

BDF_1 female mice were used to study the effects of lead acetate on macrophage activity (Blakley and Archer, 1981). Concentrations of lead acetate ranged from 50 to 1000 p.p.m. and were administered in drinking water for three weeks. Suppression of macrophage activity by lead exposure was observed when macrophage-dependent antigens, SRBC, or dinitrophenyl-Ficoll was used; however,

when the macrophage-independent antigen E. coli LPS was used, no such suppression was seen (Blakley and Archer, 1981). There were no signs of lead toxicity expressed by the mice. Results of this study indicated that lead exposure suppressed the immune response by compromising early macrophage function. The specific effect has yet to be identified but may involve recognition of the antigen, antigen processing, or amplification of the immune response (Blakley and Archer, 1981).

Phagocytosis and oxidative metabolism of cadmium and lead by peritoneal macrophages was studied by Hilbertz *et al.* (1986). Oxidative metabolism induced by phorbol myristate acetate (PMA) solution was enhanced by exposure to both metals; however, zymosan-induced oxidative metabolism was decreased significantly by cadmium exposure (and to a lesser extent by lead exposure) within the first hour of exposure (Hilbertz *et al.*, 1986). After 20 hours, lead exposure suppressed oxidative metabolism induced by zymosan and PMA along with the phagocytosis of latex-particles in a concentration-dependent manner. Lead exposure reduced cell viability only slightly (Hilbertz *et al.*, 1986). These results suggest that the enhancement of PMA-induced oxidative metabolism in the first hour of treatment with lead or cadmium results in increased release of toxic oxygen metabolites into the surrounding environment of the cell, thus impairing the defense mechanisms of macrophages (Hilbertz *et al.*, 1986).

Because lead has been found to be an immunosuppressant, Sin and Woo (1992) wanted to determine whether lead exerted a detrimental effect on phagocytic functions under acute inflammatory conditions. Female Swiss albino mice were given 2000

p.p.m. lead nitrate in their drinking water for three months. Two months after initial lead exposure the mice were vaccinated with formalin-killed parasites, Trypanosoma evansi. One month after immunization the mice were infected with live T. evansi parasites. The results suggested lead-treated mice were not immunologically protected from T. evansi infection after immunization. The total number of phagocytes in the lead-treated mice was significantly reduced compared to controls, suggesting a reduction in the total number of exudate leukocytes. This study also demonstrated lead-treatment to be detrimental to the nonspecific immune response by decreasing the chemotactic response of neutrophilic polymorphonuclear leukocytes (PMN) to inflammation. Lead may affect mononuclear phagocytes as well, causing the host to be more susceptible to infection (Sin and Woo, 1992).

Lawrence (1981) discussed the ability of heavy metals to reduce humoral immunity, cell-mediated immunity, and lymphocyte division. However, Lawrence (1981) describes lead and nickel demonstrating immunopotentiating effects by enhancing the maturation of B cells into plaque-forming cells (PFC). This effect was greater than the effect upon the development of mixed lymphocyte cultures (MLC) (Lawrence, 1981). Lead exposure was shown to enhance the MLC response of CBA/J spleen cells to C57Bl/6 stimulators, thus the ability of lead exposure to enhance the PFC response may be caused by the enhanced production of T helper cells (Lawrence, 1981). Lawrence demonstrated that T-dependent and T-independent responses were equally enhanced by lead exposure. Immunopotentiating effects of lead and nickel may be due to modulation of B cell, T cell, or macrophage functions (Lawrence,

1981).

Previously heavy metals have been shown to decrease antibody production, TcR recognition to antigen, and macrophage activity; however only recently metal toxicity and autoimmune response has been examined. Kowolenko *et al.* (1992) hypothesized that heavy metal toxicity may be due in part to autoimmunity. Heavy metals have been shown to directly modulate lymphocyte function leading to enhanced B and T cell activity which may result in autoimmunity (Kowolenko *et al.*, 1992). One particular heavy metal, lead, has been shown to affect the function of $CD4^+$ T cell subset which regulates all immune responses (McCabe and Lawrence, 1990). Lead was also found to inhibit T helper 1 cell function but to enhance T helper 2 cell function. These two subpopulations of cells (T_h 1 and T_h 2) function to regulate cell-mediated and humoral immunity (Kowolenko *et al.*, 1992). Heavy metals are also believed to be inducers of allergic hypersensitivity (Kowolenko *et al.*, 1992). Type I, II, and III hypersensitivity responses involve the production of antibody, while type IV involves a cell-mediated response. All hypersensitivity responses are activated when lymphocytes are sensitized by internal or external factors (Kowolenko *et al.*, 1992). Toxicants can cause a direct effect on the immune system by changing the lymphocyte subset ratio, changing the morphology of the immune tissue, reducing the total number of immune cells, or by changing the function of the immune system with or without the above alterations (Luster *et al.*, 1984). Toxic substances may cause tissue damage resulting in the release of sequestered antigens or an increased number of tissue antigens, thereby stimulating the immune system and causing autoimmunity

(Kowolenko *et al.*, 1992). In addition, toxicants depress immune cells, causing an increased incidence of infections and possibly an increase in autoimmune responses (Kowolenko *et al.*, 1992).

Immunoassays

Although a number of immunoassays have been developed during the last ten years, only a few have been adapted to evaluate the effects of toxicants on the immune system. Three main assays have been used to evaluate the humoral immune response: the antibody-forming cell response to T-dependent SRBC antigens; the proliferative response to B cell mitogen LPS; and the quantification of the number of B cells in the spleen (White, 1992). Measurement of the antibody-forming cell response has proven very useful, as it is a good indicator of general immunocompetence. Methods used to determine antibody-forming cells include enzyme-linked immunospot, filter immuno-plaque assay, and to measure antibody production enzyme-linked immunosorbent assay (ELISA) is used. The antibody-forming cell assay is sufficient in recognizing compounds which modulate the immune response because it is holistic in nature (White, 1992). There are also a few assays which have been used to determine the effects of toxicants on cell-mediated immunity: the mixed leukocyte response (MLR); the spleen lymphocyte response to T lymphocyte mitogen Con A; the cytotoxic T lymphocyte assay (CTL); and the delayed hypersensitivity response (DHR) to keyhole limpet haemocyanin (KLH) (White, 1992). The National Toxicology Program (NTP) also has selected assays to study natural killer (NK) cell functions and macrophage

activity (White, 1992).

Lead

Lead, an inorganic heavy metal, is a naturally occurring element which has been redistributed by humans. A large number of people and animals are exposed to lead on a daily basis, as lead is found in air, soil, paint, plastic, water, food, products of burnt coal, and batteries. Humans and animals are exposed to lead via inhalation and ingestion (Faith *et al.*, 1979). Lead poisoning has been documented when contaminated foods and beverages were consumed, when plumbing fixtures made of lead have been used in homes, and when cooking utensils containing lead were used to prepare food (Faith *et al.*, 1979). Because it causes extensive CNS damage (Goyer, 1991), lead poisoning is a major threat to the health of young children and infants. A variety of studies also have shown that lead suppresses the immune system. Depression of the activity of the immune system by lead exposure occurs at low subclinical doses and is therefore considered to be deleterious to the health of animals (Faith *et al.*, 1979).

Koller (1980) suggested that studies should be conducted to determine how contaminants affect the activity of B cells, T cells, macrophages, and the interaction of these cells in both *in vitro* and *in vivo* conditions. Lead exposure is known to suppress phagocytic function, thus reducing the immune response due to impairment of antigen processing and presentation by macrophage (Exon, 1990). Lead exposure has also been shown to affect the cell-mediated response. Faith *et al.* (1979)

demonstrated impairment of the delayed-type hypersensitivity response in Sprague Dawley rats exposed to lead acetate. In an *in vitro* study of T-lymphocyte activity, CBA/J and C57Bl/6 mice infected with Listeria monocytogenes and treated with high concentrations of lead acetate showed a slight reduction in T cell activity (Lawrence, 1981).

Chagas' disease

In 1907 Carlos Chagas located an area of Brazil, Minas Geraes, where thatched roof houses were infested with blood sucking (haematophagus) bugs, Conorhinus megistus. The bugs were examined and flagellated protozoa were found in their hindguts. Flagellates could be found in the bloodstream 20-30 days after an individual was bitten by the reduviid bug. The bugs were examined at the Institute Oswaldo Cruz, thus the parasites were termed Trypanosoma cruzi (Minchin, 1910).

The causative agent of Chagas' disease is the flagellated protozoan, Trypanosoma cruzi, which has a single nucleus and undergoes three life cycle stages: (1) the intracellular amastigote stage, which is round and lacks a flagellum replicates in the mammalian host; (2) the epimastigote stage, which possesses a juxtanuclear kinetoplast and a flagellum replicates in the vector; and (3) the trypomastigote stage, which possesses a flagellum and has a post-nuclear kinetoplast does not replicate (Wendel and Gonzaga, 1993). Amastigotes are found in vertebrate macrophages, muscle fibers, and within cells of the central nervous system (CNS). Epimastigotes are seldom detected in the vertebrate bloodstream; however, this form is common in

the foregut of the reduviid bug. Trypomastigotes are located in the vertebrate bloodstream, lymph and cerebrospinal fluid, and in the invertebrate duodenum and hindgut (Wendel and Gonzaga, 1993). Trypomastigotes are approximately 20 μm long, may be short and thick or long and slender, and form a characteristic C or U shape in bloodsmears (Markell *et al.*, 1986).

Transmission

The trypomastigote is the infective stage of the parasite for humans. T. cruzi parasites are introduced via fecal material into the bitten area when a reduviid bug takes a blood meal. Infection occurs when infective trypomastigotes are rubbed into the wound or eye, or cross the mucosa. Lymph fluid transports the parasites to the lymph nodes. After the trypomastigote enters the bloodstream it invades macrophages or other nucleated cells where it transforms into the amastigote stage and divides by binary fission. The amastigote avoids degradation by escaping the phagosome and entering the cytosol of the macrophage prior to lysosomal fusion. Amastigotes transform back into trypomastigotes, which are released when the cell ruptures and are free to invade other cells or enter the circulatory system. The trypomastigote can independently penetrate nucleated cells or it may gain access by phagocytosis. The organs most susceptible to infection are spleen, liver, lymph nodes, and muscles (skeletal and cardiac). The infection also effects the nervous system, reproductive system, intestine, Kupffer cells, reticuloendothelial cells, and bone marrow (reviewed in Bogitsh and Cheng, 1990).

T. cruzi has several natural reservoirs including skunks, marmots, sloths, bats, foxes, jaguars, ferrets, rabbits, mice, hamsters, marmosets, armadillos, raccoons, opossums, monkeys, and squirrels (Wendel and Gonzaga, 1993). It has been shown that birds and reptiles are resistant to T. cruzi infection (Wendel and Gonzaga, 1993). Chagas' disease via blood transfusions is relatively frequent (200 reported cases, many go unreported) in Latin America. Infected individuals tend to live in low socioeconomic areas where blood screening is not available. Even when screening tests are performed on blood donor samples, T. cruzi has a phenomenal viability. Whole blood infected with T. cruzi can survive refrigeration for 18 days and withstand freezing for 24 hours, indicating a high risk of transmission via blood transfusions. All blood products, unless sterilized, may be infectious. The frequency of transmission through blood transfusions depends on parasite strain, parasitemia levels of donor blood, recipient's immune system, and quantity of blood transfused (Wendel and Gonzaga, 1993). Tissues used for organ transplantation can also transmit T. cruzi infection (Wendel and Gonzaga, 1993).

Reports indicate congenital transmission is possible yet uncommon. Breast feeding has also been shown to be a possible mode of transmission (Wendel and Gonzaga, 1993).

Disease symptoms and stages

Chagas' disease is most common and most severe in children under five. Symptoms occur predominantly in the central nervous system (CNS), and children may

develop meningoencephalitis and die within days. Infants experience fever, lymphadenitis, hepatosplenomegaly, and anasarca (Markell *et al.*, 1986).

Older children and adults typically have a milder, subacute, or chronic form of the disease with no CNS complications (Markell *et al*, 1986). The acute stage ends in a few weeks either in death (approximately 10% frequency) (Miles, 1983), full recovery, or transition to the chronic stage.

Following infection, the parasites enter the subcutaneous tissue macrophages where localized swelling occurs (chagoma). Facial and ocular edema is commonly unilateral (Wendel and Gonzaga, 1993). The lesion reaches full size within several days and slowly decreases size in two or three months (Markell *et al.*, 1986). Symptoms of infection appear four to fourteen days post inoculation. Parasites can be microscopically detected on day 10 of infection and persist during the acute phase. The symptoms during the acute stage of infection include fever, malaise, and headaches. Hepatosplenomegaly and myocardial damage may occur; however, cardiac involvement and gastrointestinal symptoms may not appear until many years following infection (Bogitsh and Cheng, 1990). Other clinical observations include tremors, seizures, meningitis, and lymphadenopathy (Wendel and Gonzaga, 1993). Patients with symptomatic infections may exhibit signs and symptoms of congestive heart failure (mainly in the right ventricle) but this condition is rare in patients under 25 years of age (Markell *et al.*, 1986). In early chronic stages, the heart may be normal in size or slightly larger; however, in late stages approximately 30% of infected individuals develop severe cardiomegaly (Miles, 1983). Inflammation, fibrosis, and

infiltration by lymphocytes, macrophages, and plasma cells all occur within cardiac muscle.

Immunoprotection of T. cruzi

Immunoprotective mechanisms for T. cruzi infection involve both humoral immunity and cell-mediated immunity. Immunoglobulin class IgG is the primary antibody produced during the course of infection (Markell *et al.*, 1986). Parasite lysis is achieved by: antibody-dependent and complement-mediated mechanisms; natural killer (NK) cells; and cytotoxic T lymphocytes (CTL). Activated macrophages, neutrophils, and eosinophils also destroy trypomastigotes during cell-mediated responses (Markell *et al.*, 1986; Kuhn, 1989).

Research

Over the past 15 years, research has been conducted on the immune response of lead-treated experimental animals challenged with bacterial and viral infections. However, little research has been done on the effects of lead exposure during parasitic infection. In the present study, the effect of lead acetate treatment during infection with Trypanosoma cruzi was investigated.

The present study was designed to examine the effects of high lead acetate concentrations on the humoral immune response of C57Bl/6 female mice when challenged with T. cruzi. Same sexed animals were used in this study to eliminate gender variability. C57Bl/6 mice are not highly susceptible to the Brazil strain of T.

cruzi and normally live 46-180 days post infection with parasitemias peaking at 7.5 x 10^5 parasites/ml of blood (Grogl and Kuhn, 1985). C57Bl/6 mice were chosen for this study in order to better demonstrate whether lead acetate showed humoral immunosuppressive qualities. The specific questions addressed in this study included: 1) What is the effect of lead acetate on parasitemia levels?; 2) What is the effect of lead acetate on the mortality rate?; 3) How does lead acetate affect parasite-specific antibody production?; and 4) Does exposure to lead acetate affect the growth rate of mice infected with T. cruzi?

MATERIALS AND METHODS

Mice

Seventy-two C57Bl/6 female mice were obtained at 5 weeks of age (Jackson Laboratory, Bar Harbor, Maine) and acclimated for two weeks on deionized water and Laboratory Rodent Chow (PMI Feeds, St. Louis, Missouri). Mice were then divided into three replicate trials containing 24 mice, and each replicate consisted of four experimental groups with six mice per group. The four experimental groups were given the following lead acetate concentrations in their drinking water for three weeks prior to infection: Group 1-0 p.p.m., Group 2-10 p.p.m., Group 3-100 p.p.m., and Group 4-1000 p.p.m. After three weeks of lead exposure, mice received an intraperitoneal injection of $1x10^4$ blood-form trypomastigotes (BFTs) of Brazil strain Trypanosoma cruzi, which is continuously maintained in the laboratory as a stock infection in C3HeB/FeJ mice. Mice in replicates II and III were inoculated with a different isolate of the same Brazil strain of T. cruzi as used for mice in replicate I.

Lead acetate (Sigma Chemical Company, St. Louis, Missouri), 0.002 g, was dissolved in 2 L of deionized water to make a 1000 p.p.m. stock solution. The remaining concentrations were diluted from the stock.

H_2O and food was provided *ad libitum* and levels were measured during the study. At two week intervals, the weight (g) of each mouse was measured and

recorded.

Preparation of T. cruzi antigen extract

A PSC3H murine fibroblast cell line infected with T. cruzi trypomastigotes was cultured in RPMI-1640 medium (Sigma) supplemented with 10% newborn calf serum (Microbiological Associates, Walkersville, Maryland), 25 mM HEPES (Sigma), and penicillin/streptomycin. Culture supernatants containing typomastigote stages were collected and filtered through Whatman #1 filter paper. The filtered supernatant was then centrifuged for 30 minutes at 1800 x g. The pellet, which contains parasites, was resuspended in 1 ml of Dulbecco's Phosphate Buffered Saline (DPBS), placed in a 1.7 ml Eppendorf tube (Fisher Scientific, Pittsburgh, Pennsylvania), and centrifuged for 15 minutes at 2000 x g. The pellets were resuspended two more times, combined and resuspended in ice-cold DPBS containing 0.5% Triton X and 2 mM phenylmethylsulfonyl fluoride (Sigma) and the suspension was transferred to a 15 ml centrifuge tube and incubated for 30 minutes in an ice bath while being vortexed periodically. The resulting suspension was spun at 8,000 rpm for 15 minutes to remove insoluble material; the supernatant was collected. A Bio-Rad protein determination assay was performed on the antigen extract using bovine serum albumin as a standard. The antigen extract was diluted to a working concentration of 50 µg/ml in DPBS for use in an indirect enzyme linked immunosorbent assay (ELISA).

Preparation of murine plasma

At two week intervals during the acute stage of infection (14-50 days), 4 µl of tail vein blood was extracted from each of the 72 mice. Blood was transferred to 1.7 ml Eppendorf tubes, and 96 µl of DPBS were added to each sample. The solution was mixed thoroughly then centrifuged at 5,000 rpm for 10 minutes. Plasma samples were stored at -4^0 C until used in indirect ELISA assays.

Parasitemia

Parasite counts were conducted at 3 and 4 day intervals starting at day 14 post infection. Four µl of tail vein blood was mixed with 96 µl DPBS in Eppendorf tubes, constituting a 1/25 dilution. A hemacytometer (Fisher Scientific) was used to count the parasites by placing approximately 20 µl of the cell suspension on the slide and counting the parasites seen in the four large corner squares. The number of cells/ml of blood was calculated using the equation: cells/ml = (avg. number/large square) * 10^4/ml * 1/dilution. Parasitemias were conducted until the levels declined to near zero.

Mean peak parasitemias were calculated by determining the peak parasitemia for each treatment group for each day analyzed. The three replicate experiments (I, II, and III) were combined by calculating the mean peak of all mice receiving each lead acetate concentration.

Indirect ELISA

Individual wells of 96 well microtiter plates (Falcon) were incubated with 100

µl of a T. cruzi culture cell antigen extract (50 µg/ml in DPBS). Plates were incubated at room temperature overnight and then stored at -70° C until use. Thawed plates were rinsed three times with fresh DPBS, then blotted dry. Wells were filled with blocking solution (2.5% Carnation Instant Nonfat Dry Milk in DPBS) and incubated for one hour at 37° C. After incubation, plates were rinsed three times and blotted dry. One hundred µl of 1:25 diluted murine plasma was placed into the first well of each row of the microtiter plate and a 2 x serial dilution of the plasma in DPBS was performed across the plate. Each sample of murine plasma was tested in duplicate rows. Plates were covered and incubated for two hours at 37° C. After incubation, plates were washed four times with DPBS. After the fourth wash wells were filled with DPBS and incubated for 5 minutes. Once plates were blotted dry, 50 µl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma) diluted 1:1000 in blocking solution was added to each well. Plates were covered and incubated for one hour at 37° C. After incubation the plates were washed four times with DPBS and blotted dry with paper towels. Fifty microliters of substrate solution were added to each well and incubated for 15 minutes at 37° C. Substrate solution was prepared by dissolving 30 mg O-phenylenediamine dihydrochloride (OPD; Sigma) in 10 ml Tris Citrate Buffer (0.3 M Tris base in 0.5% Triton X-100 pH to 6.0 with anhydrous citric acid). Eight µl of 0.025% H_2O_2 was added to the solution just prior to adding the substrate to the wells. Plates were analyzed on a Model 550 microplate reader (Bio-Rad) at an absorbance of 490 nm. Antibody titers were determined according to the following equation: titer = highest dilution of the test serum in which

the absorbance is > .05 and > the mean control absorbance +2 standard deviations.

Statistical analysis

Data were analyzed using SYSTAT@ 5.0 (SYSTAT, Inc., 1992). Analysis of variance (ANOVA) was used to test for differences in parasitemia counts among lead-treated groups within individual replicate experiments. To determine which treatment groups differed significantly, a pairwise comparison using Tukey's HSD Multiple Comparison Test was employed (SYSTAT Inc., 1992).

To evaluate significant correlation between parasitemia and antibody production a Spearman correlation coefficient was used (Wilkinson, 1992).

To examine potential changes in food and water consumption over time, a linear regression analysis of slopes was conducted. Slopes of zero suggest each lead-treatment group per cage maintained the same diet patterns throughout the study. An analysis of covariance (ANCOVA) was used to test for significance among treatment groups' diet patterns in each replicate experiment. The assumption of homogeneity of slopes was by Multivariate General Linear Hypothesis (MGLH) and when satisfied ANCOVA, was employed to test for significance among lead-treated groups. To determine which treatment groups differed significantly a pairwise comparison using Tukey's HSD Multiple Comparison Test was employed (SYSTAT Inc., 1992).

Testing for significant changes in mouse weight was conducted by using ANCOVA. MGLH and Tukey's HSD test were used as described above.

RESULTS

Mortality

Mortality was low during the course of the present study; however, one mouse in replicate I receiving 1000 p.p.m. lead acetate concentrations died on day 63 post infection.

Mouse weights

In all three replicate experiments weight changed significantly over time; I-β=0.019, $p<0.001$, II-β=0.025, $p<0.001$, and III-β=0.025, $p<0.001$ (Figure 1).

The assumption of homogeneity of slopes was satisfied for all lead-treatment groups in replicate I ($F_{3,1}$=0.773, p=0.510). Tests of intercept differences among treatment groups were significant ($F_{3,1}$=3.766, p=0.012). Pairwise comparisons of all lead-treatment groups indicated that mice receiving 100 p.p.m. lead acetate weighed significantly more than mice receiving 1000 p.p.m. (p=0.009) (Figure 1a).

The assumption of homogeneity of slopes was satisfied for all lead-treatment groups in replicates II and III ($F_{3,1}$=0.953, p=0.418 and $F_{3,1}$=0.354, p=0.787). However, ANCOVA analysis for intercept differences in replicates II and III were nonsignificant ($F_{3,1}$=0.984, p=0.403 and $F_{3,1}$=2.371, p=0.076, respectively) (Figures 1b and 1c).

Water and food consumption

Replicate by treatment combinations showed 10 of 12 regressions of average daily water consumption over time were nonsignificant. Mice receiving 10 p.p.m. ($p=0.023$) and 1000 p.p.m. ($p=0.001$) lead acetate concentrations in replicate I showed significant decrease in water consumption over time (Figure 2a). However, when data from all treatments are pooled ANCOVA showed there were significant decreases in water consumption over time in all three replicates (I-$F_{3,1}=16.750$, $p<0.001$, II-$F_{3,1}=10.522$, $p=0.002$, III-$F_{3,1}=8.006$, $p=0.006$) (Figure 2). Nevertheless, average daily water consumption among groups in each of the three replicates showed homogeneity of slopes ($F_{3,1}=1.573$, $p=0.201$;$F_{3,1}=9.596$, $p=0.898$ and $F_{3,1}=4.425$, $p=0.940$, respectively). ANCOVA analysis showed significant differences among treatment groups in replicate I ($F_{3,1}=4.672$, $p=0.004$). Mice receiving 100 p.p.m. lead acetate concentrations drank significantly more water on average than did mice receiving 10 or 1000 p.p.m. lead acetate concentrations ($p=0.038$ and $p=0.004$, respectively) (Figure 2a). There were no statistical differences in among treatment groups in replicates II and III ($F_{3,1}=1.075$, $p=0.364$; $F_{3,1}=0.020$, $p=0.996$) (Figure 2c).

Replicate by treatment combinations showed 11 of 12 regressions of average daily food consumption over time were nonsignificant. One slope from Group 4-1000 p.p.m. ($p=0.022$) in replicate I showed significant decrease in food consumption over time (Figure 3a). However, when data from all treatments are pooled ANCOVA showed there were significant decreases in food consumption over time in replicates I and III (I-$p=0.007$ and III-$p=0.001$) (Figures 3a and 3c). The average daily food

consumption in replicates I, II, and III showed homogeneity of slopes among treatment groups ($F_{3,1}$=0.393, p=0.758), ($F_{3,1}$=0.004, p=1.000) and ($F_{3,1}$=0.035, p=0.991); however, ANCOVA showed no statistical differences among lead-treatment groups (I-$F_{3,1}$=2.114, p=0.114, II-$F_{3,1}$=0.436, p=0.728, and III-$F_{3,1}$=0.463, p=0.709) (Figure 3).

Parasitemia

Overall mean peak parasitemias of each treatment group receiving lead acetate are as follows: 0 p.p.m. lead acetate (infected control mice)-6.49 x 10^6 parasites/ml blood; 10 p.p.m. lead acetate-9.61 x 10^6 parasites/ml blood; 100 p.p.m. lead acetate-1.31 x 10^7 parasites/ml blood (twofold higher than infected control mice); and 1000 p.p.m. lead acetate-3.62 x 10^7 parasites/ml blood (sixfold higher than infected control mice) (Figure 4).

The highest numbers of circulating blood-form trypomastigotes in all replicates were observed in lead-treated mice receiving 100 and 1000 p.p.m. lead acetate concentrations (Figure 5). Replicate I showed extremely high parasitemias (7.1 x 10^7 parasites/ml of blood) on day 39 of infection in mice receiving 1000 p.p.m. lead acetate (Figure 5a). An analysis of variance of parasitemias from day 39 post infection was highly significant ($F_{3,1}$=30.470, p<0.001); and HSD test showed Group 4-1000 p.p.m. had significantly higher parasitemias than Groups 1-0 p.p.m. (p<0.001), 2-10 p.p.m. (p<0.001), or 3-100 p.p.m. (p<0.001) (Figure 5a).

The highest peak parasitemia in replicate II was 9 x 10^6 parasites/ml of blood observed on day 28 of infection in mice receiving 100 p.p.m. lead acetate (Figure 5b).

Mice receiving 1000 p.p.m. lead acetate in replicate II showed two peaks; on day 21 of infection, mice had an average of 6.0 x 10^6 parasites/ml of blood, and on day 28 the average was 6.5 x 10^6 parasites/ml of blood (Figure 5b). An analysis of variance showed no statistical differences among lead-treatment groups on day 21 ($F_{3,1}$=1.515, p=0.241); however, parasite levels on day 28 differed significantly ($F_{3,1}$=5.263, p=0.008), as Group 3 showed significantly higher parasitemias than Group 1 (p=0.004) (Figure 5b).

Mice receiving 100 p.p.m. lead acetate in replicate III showed two peaks in parasitemias. On day 14 of infection, mice had an average of 1.4 x 10^7 parasites/ml of blood, and on day 21 the average was 1.8 x 10^7 parasites/ml of blood (Figure 5c). Mice receiving 1000 p.p.m. lead acetate concentrations in replicate III showed their peak parasitemia (1.8 x 10^7 parasites/ml of blood) on day 14 of infection (Figure 5c). An analysis of variance of parasitemias on day 14 was highly significant ($F_{3,1}$=5.849, p=0.005); Group 4 had significantly higher parasitemias than Group 1 (p=0.003) (Figure 5c). By contrast, on day 21 post infection, ($F_{3,1}$=5.797, p=0.005) showed Group 1 had significantly higher parasitemias than Group 4 (p=0.004) based on ANOVA and HSD tests (Figure 5c).

Parasite-specific antibody levels

The parasite-specific antibody production did not show an obvious relationship between high lead acetate concentrations (100 and 1000 p.p.m.) and decreased antibody production throughout infection in serum samples diluted 1:25 (Figure 6).

This dilution was chosen for analysis to demonstrate antibody production at the lowest dilution factor. Spearman correlation coefficient testing showed there was not a significant correlation between antibody production and parasitemia ($p=0.252$). Parasite-specific antibody production in mice receiving 100 and 1000 p.p.m. lead acetate concentrations from replicate I was high on day 32 of infection and low on days 18 and 46 of infection (Figure 6a). Infected control mice receiving 0 p.p.m. lead acetate showed their peak anti-T. cruzi antibody production on day 46 post infection (Figure 6a).

Parasite-specific antibody production in mice receiving 100 p.p.m. lead acetate from replicate II was highest on days 21 and 51 of infection and low on day 35 (Figure 6b). Mice receiving 1000 p.p.m. lead acetate in replicate II showed low antibody production on days 21 and 35 of infection and high production on day 51 (Figure 6b). Infected control mice receiving 0 p.p.m. lead acetate showed their peak anti-T. cruzi antibody production on day 51 post infection (Figure 6b).

In replicate III the parasite-specific antibody production in mice receiving 100 and 1000 p.p.m. lead acetate was highest on day 41 of infection and lowest on day 14 (Figure 6c). Infected control mice treated with 0 p.p.m. lead acetate showed their peak anti-T. cruzi antibody production on day 30 post infection (Figure 6c). Anti-T. cruzi antibody titers for all replicate experiments are shown in Table 1.

Table 1. The titers of anti-T.cruzi antibodies in mice receiving 0, 10, 100, and 1000 p.p.m. lead acetate concentrations in a) Replicate I, b) Replicate II, and c) Replicate III.

a.

lead acetate concentrations	day 18	day 32	day 46
0 p.p.m.	1:25600	1:800	1:400
10 p.p.m.	1:1600	1:800	1:6400
100 p.p.m.	1:6400	1:800	1:3200
1000 p.p.m.	1:800	1:1600	1:6400

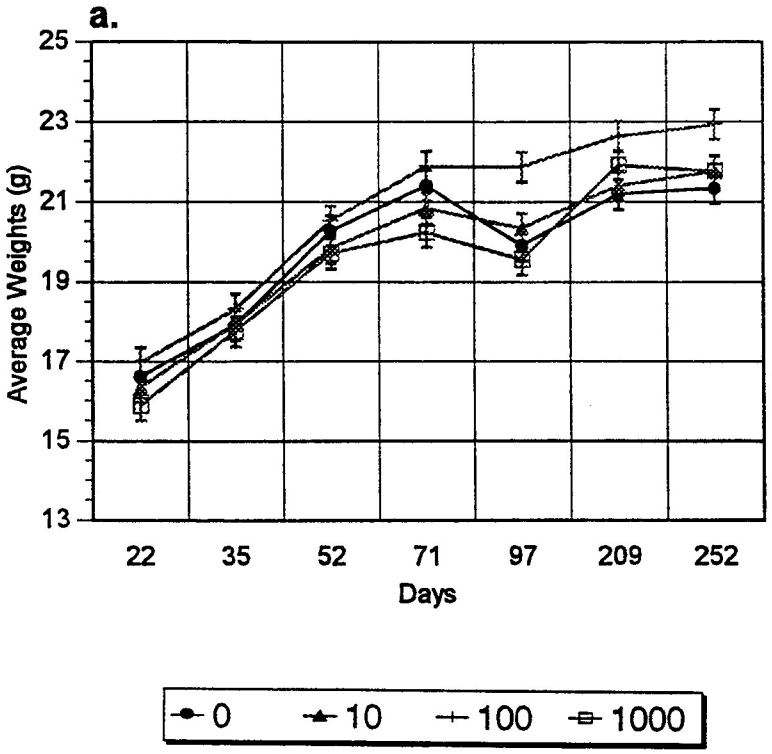
b.

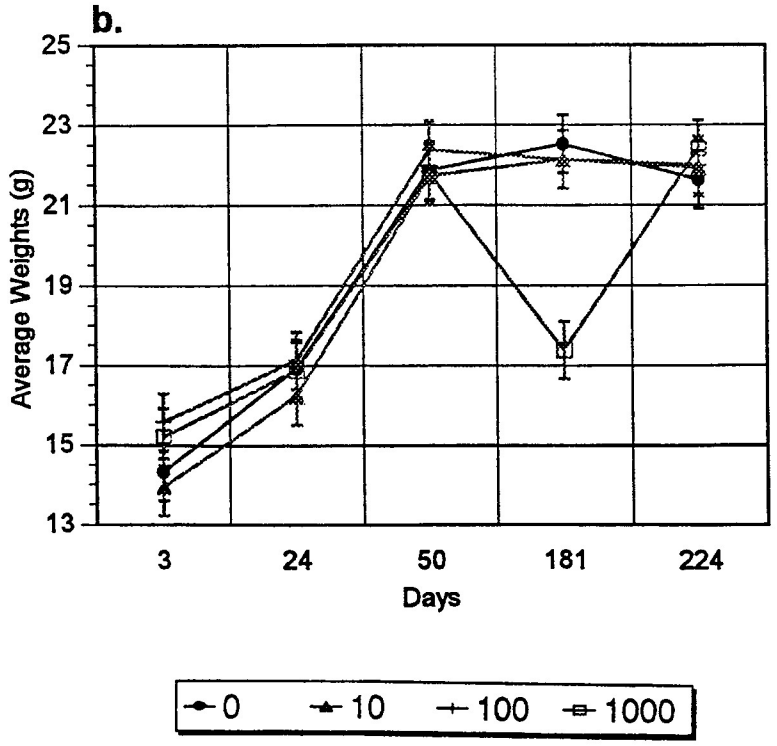
lead acetate concentrations	day 21	day 35	day 51
0 p.p.m.	1:400	1:50	1:1600
10 p.p.m.	1:800	1:50	1:800
100 p.p.m.	1:800	1:1600	1:1600
1000 p.p.m.	1:100	1:800	1:800

c.

lead acetate concentrations	day 14	day 30	day 41
0 p.p.m.	1:400	1:6400	1:3200
10 p.p.m.	1:400	1:51200	1:1600
100 p.p.m.	1:400	1:1600	1:3200
1000 p.p.m.	1:800	1:800	1:1600

FIGURE 1. Average weights of mice measured over the course of the study before and after lead exposure and T. cruzi infection in a) Replicate I, b) Replicate II, and c) Replicate III. Results are presented $\pm$ 1 SE bar to suggest significance among treatment groups.


a.
25
23
21
19
17
15
13
Average Weights (g)
22
35
52
71
97
209
252
Days
0
10
100
1000


b.
25
23
21
19
17
15
13
Average Weights (g)
3
24
50
181
224
Days
0
10
100
1000

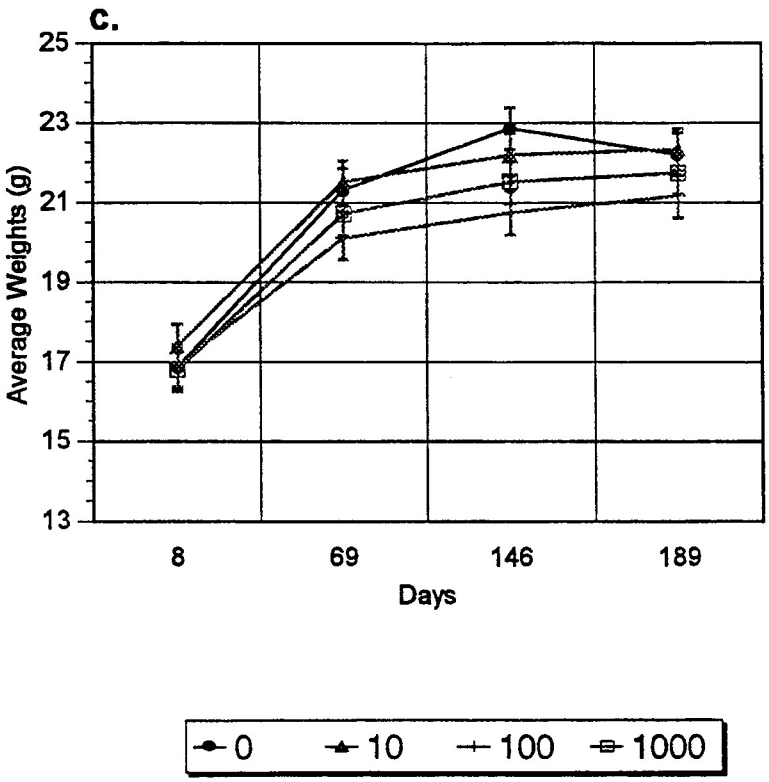
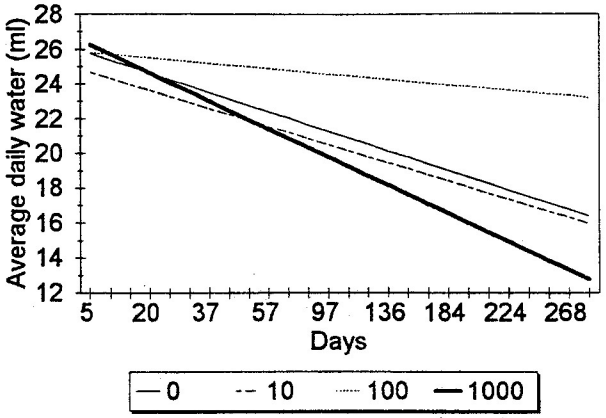
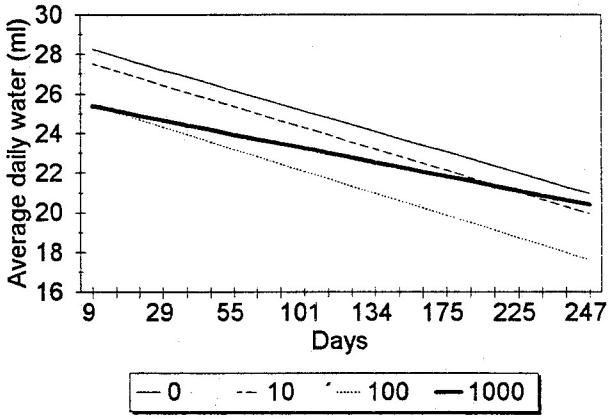

C.
25
23
21
19
17
15
13
Average Weights (g)
8
69
146
189
Days
0
10
100
1000

FIGURE 2. Linear regression analysis of average daily water consumption per cage measured over the course of the experiment in a) Replicate I, b) Replicate II, and c) Replicate III.

a.


Average daily water (ml)
28
26
24
22
20
18
16
14
12
5 20 37 57 97 136 184 224 268
Days
0 10 100 1000

b.


Average daily water (ml)
30
28
26
24
22
20
18
16
9 29 55 101 134 175 225 247
Days
0 10 100 1000

c.

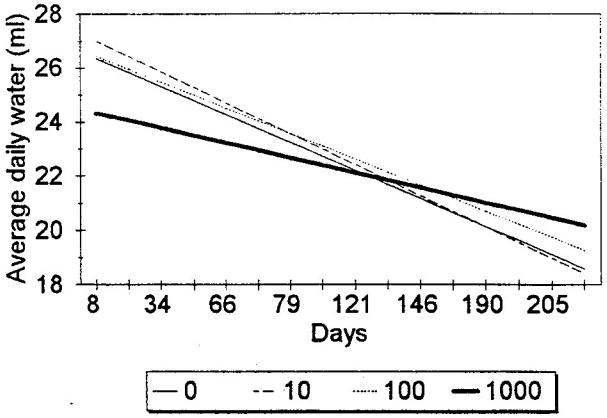
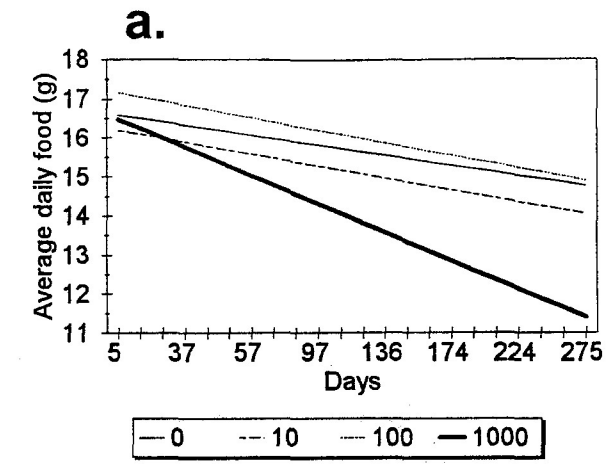
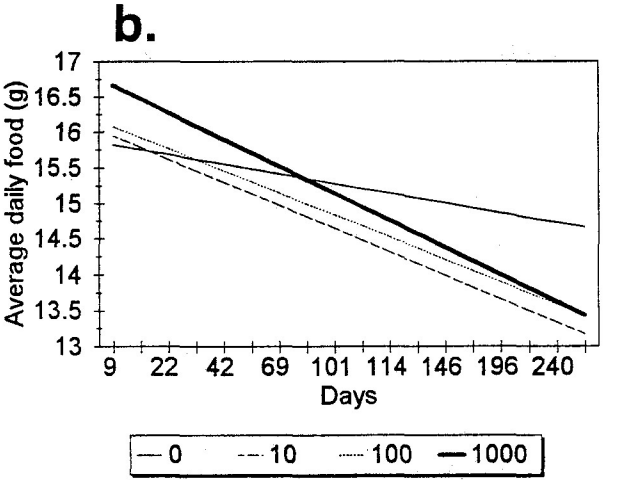

Average daily water (ml)
28
26
24
22
20
18
8 34 66 79 121 146 190 205
Days
0 10 100 1000

FIGURE 3. Linear regression analysis of average daily food consumption per cage measured over the course of the experiment in a) Replicate I, b) Replicate II, and c) Replicate III.


a.
Average daily food (g)
18
17
16
15
14
13
12
11
5
37
57
97
136
174
224
275
Days
0
10
100
1000


b.
Average daily food (g)
17
16.5
16
15.5
15
14.5
14
13.5
13
9
22
42
69
101
114
146
196
240
Days
0
10
100
1000

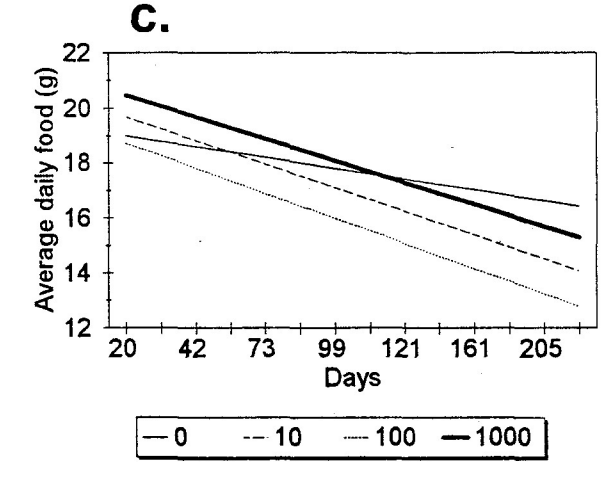
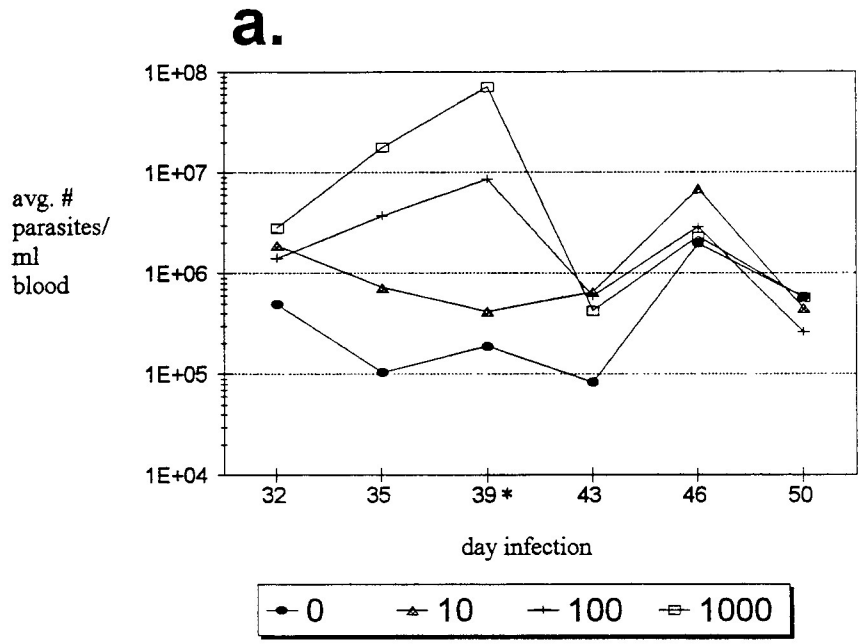
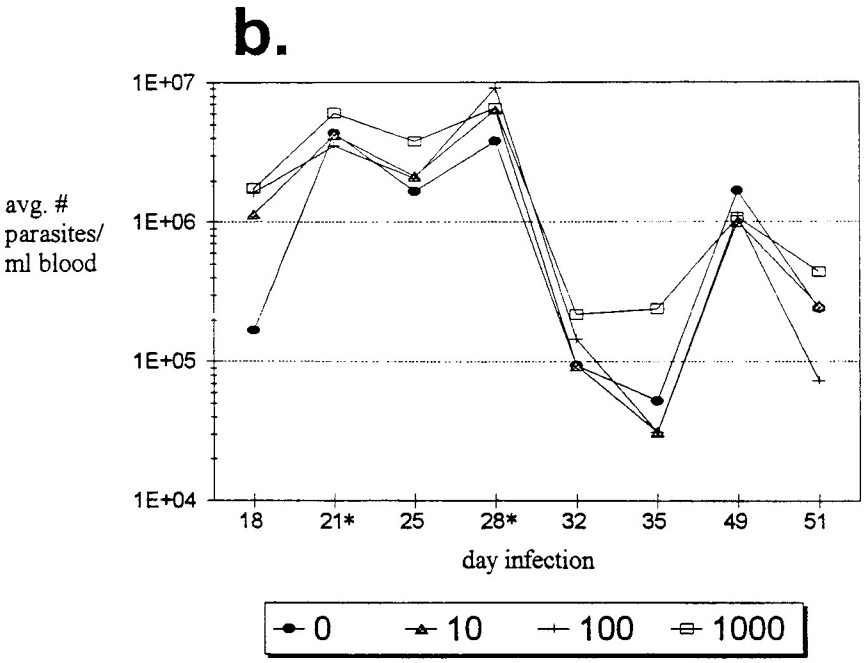

c.
Average daily food (g)
22
20
18
16
14
12
20
42
73
99
121
161
205
Days
0
10
100
1000

FIGURE 4. Mean peak parasitemias ± 1 SE of mice receiving 0, 10, 100, and 1000 p.p.m. lead acetate concentrations. The peak parasitemia was determined for each treatment group for each day analyzed and an average was calculated. All three replicate experiments were combined to obtain an overall mean peak parasitemia for each lead acetate concentration (n=18 for all groups).


a.
avg. # parasites/ ml blood
1E+08
1E+07
1E+06
1E+05
1E+04
32
35
39*
43
46
50
day infection
0
10
100
1000


b.
avg. # parasites/ ml blood
1E+07
1E+06
1E+05
1E+04
18
21*
25
28*
32
35
49
51
day infection
0
10
100
1000

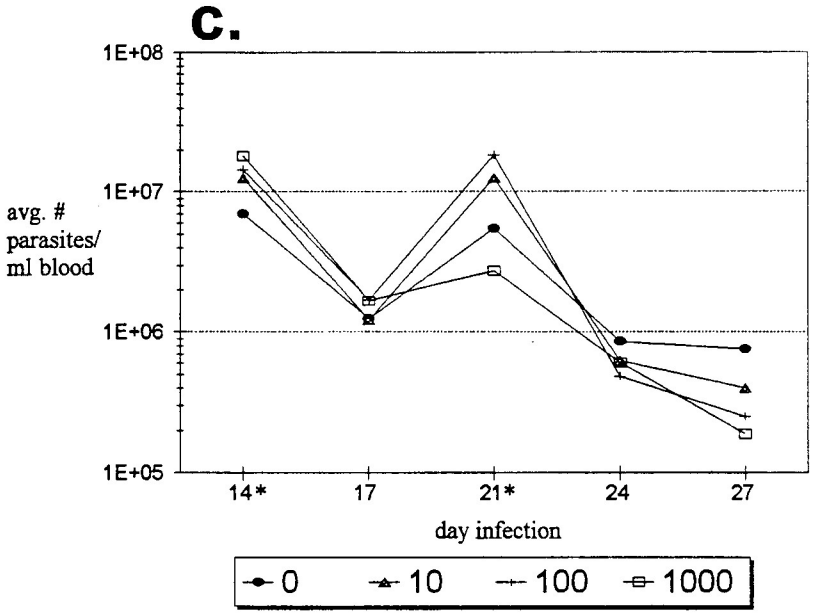

C.
1E+08
1E+07
1E+06
1E+05
avg. # parasites/ ml blood
14*
17
21*
24
27
day infection
0
10
100
1000

FIGURE 5. Mean parasitemias per ml of blood from mice receiving 0, 10, 100, and 1000 p.p.m. lead acetate concentrations during infection with T. cruzi. Data are expressed logarithmically as the average number of parasites per ml of blood in a) Replicate I, b) Replicate II, and c) Replicate III. (*) represents days analyzed by ANOVA.

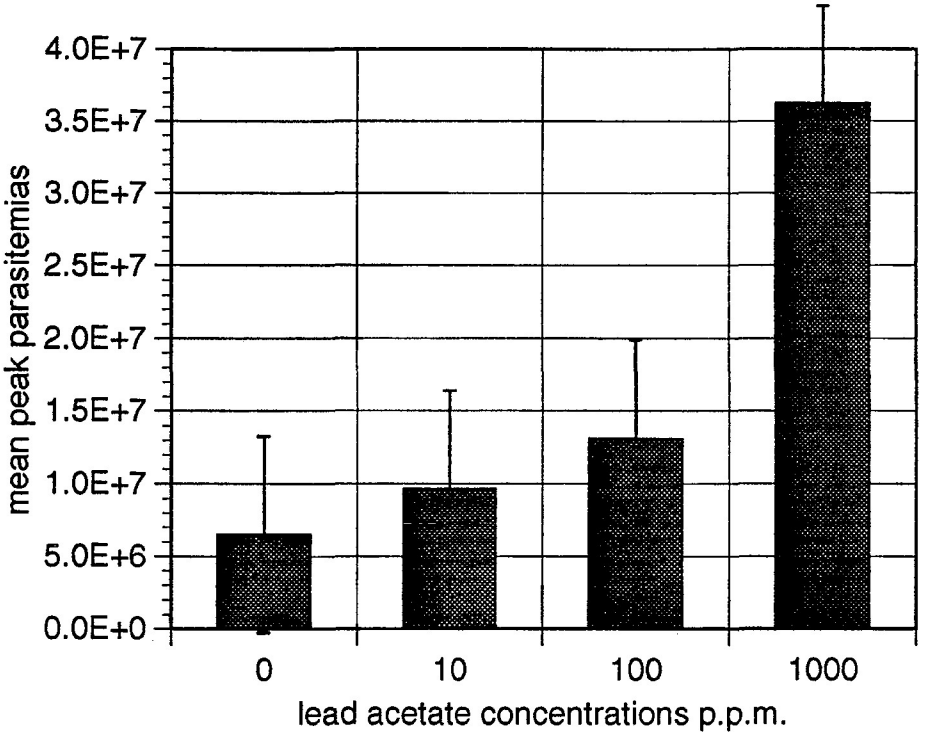
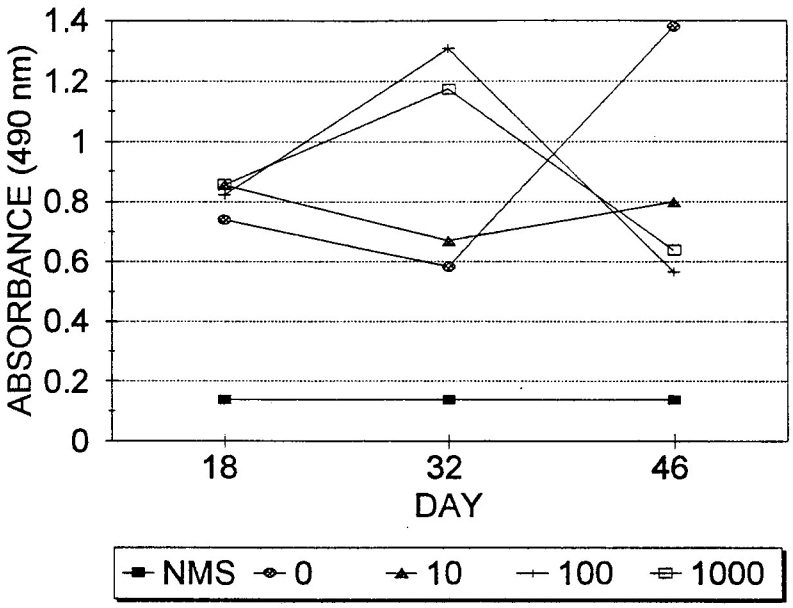
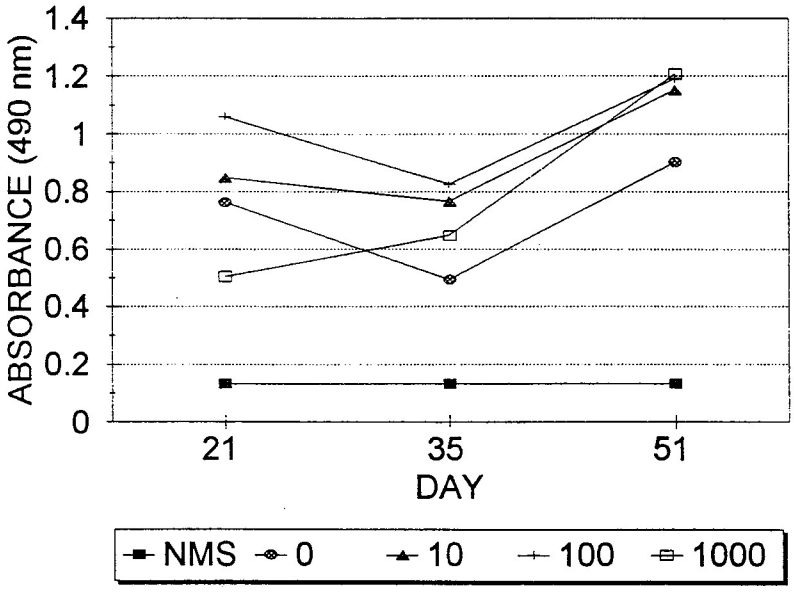

4.0E+7
3.5E+7
3.0E+7
2.5E+7
2.0E+7
1.5E+7
1.0E+7
5.0E+6
0.0E+0
mean peak parasitemias
0
10
100
1000
lead acetate concentrations p.p.m.

FIGURE 6. Parasite-specific antibody levels in mice exposed to 0, 10, 100, and 1000 p.p.m. lead acetate concentrations measured from pooled serum samples in a) Replicate I, b) Replicate II, and c) Replicate III. Data presented represent absorbances of serum samples diluted 1:25 and read at 490 nm. For each lead acetate concentration n=6 for all replicates. NMS-normal mouse serum (control) (n=2).

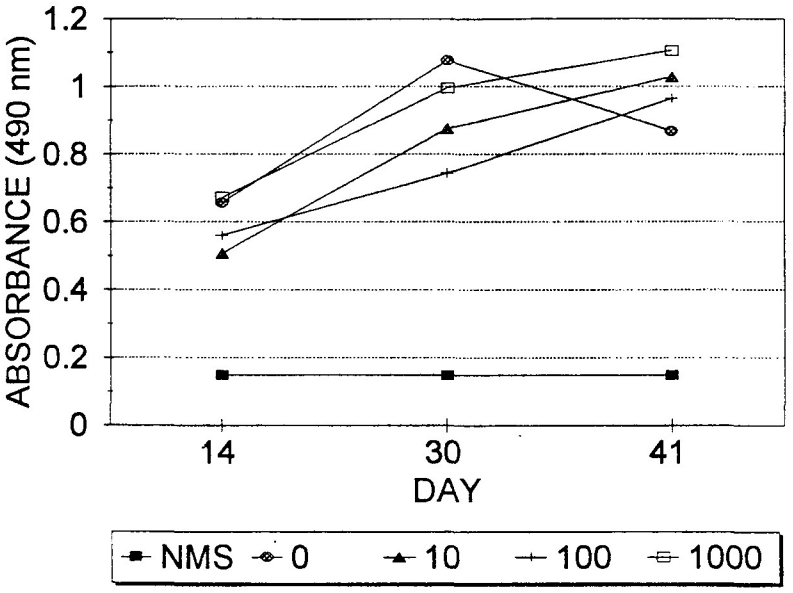
a.


ABSORBANCE (490 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
18
32
46
DAY
NMS
0
10
100
1000

b.


ABSORBANCE (490 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
21
35
51
DAY
NMS
0
10
100
1000

c.



DISCUSSION

The results of the present research confirm previous studies which have suggested that lead acetate possesses immunosuppressive qualities which may increase host susceptibility to infectious disease. However, this research project is the first that has evaluated the effect of exposure to lead acetate during experimental Chagas' disease.

The results of the present study suggest exposure to high lead acetate concentrations does increase the susceptibility of C57Bl/6 mice challenged with the Brazil strain of Trypanosoma cruzi. The greatest impact of high lead acetate exposure was on numbers of circulating blood-form trypomastigotes (BFTs). C57Bl/6 mice infected with the Brazil strain of T. cruzi normally exhibit only moderate parasitemias and a limited degree of histopathology (Rowland *et al.*, 1992; Sun and Tarleton, 1993). However, in the present study mice exposed to 100 and 1000 p.p.m. lead acetate experienced extremely high numbers of circulating BFTs, which is generally regarded as a direct indication of extensive tissue parasitism (Lima *et al.*, 1995) and disease severity. Rowland *et al.* (1992) reported that pathopermissive mouse strains (strains exhibiting a high degree of histopathology) have a much higher parasite load than pathoresistant strains. The high parasite loads observed in lead-treated mice in the present study suggest that lead-acetate may suppress the immune response to T. cruzi.

Tarleton (1990) demonstrated C57Bl/6 mice immunosuppressed (depleted of $CD8^+$ T cells) prior to infection with T. cruzi had higher parasitemias and increased disease severity as compared to nonsuppressed control mice. Furthermore, immunosuppressed individuals usually experience increased parasitemias, localized tissue parasites, increased mortality, and increased severity of symptoms of Chagas' disease (Markell *et al.*, 1986; Calabrese *et al.*, 1991).

Highest observed numbers of circulating BFTs were detected in mice receiving 100 and 1000 p.p.m. lead acetate concentrations in all three replicate experiments. Mean peak parasitemias calculated from mice receiving a lead acetate concentration of 1000 p.p.m. (3.62×10^7 parasites/ml of blood) were sixfold higher than in 0 p.p.m. lead acetate-treated mice (6.49×10^6 parasites/ml of blood) (Figure 4). Also, mean peak parasitemias calculated from mice receiving 1000 p.p.m. lead acetate showed no overlap of SE bars when compared to other treatment groups (Figure 4). No statistical analysis was conducted on mean peak parasitemia data because different isolates were used in replicates II and III, and parasitemia counts were not conducted on the same days post infection. However, these results strongly suggest there is a dose dependent effect of lead acetate resulting in increasing numbers of circulating BFTs in treated mice.

Mice receiving 1000 p.p.m. lead acetate concentrations in replicate experiment I showed a peak of 7.1×10^7 parasites/ml of blood on day 39 post infection which was 374 times greater than observed circulating BFTs in mice receiving 0 p.p.m. lead acetate (1.9×10^5 parasites/ml of blood) (Figure 5). In replicate experiment II, mice

receiving 100 p.p.m. lead acetate had two times more parasites/ml of blood on day 28 post infection (9.0×10^6) than that observed in mice receiving 0 p.p.m. (3.8×10^6 parasites/ml of blood) (Figure 5). In replicate experiment III, on day 14 post infection, mice exposed to 1000 p.p.m. lead acetate had three times more circulating BFTs than that observed in 0 p.p.m. lead-treated mice; however, on day 21 post infection, a peak of 2.7×10^6 parasites/ml of blood in mice treated with 1000 p.p.m. lead acetate concentrations was two times lower than that observed in mice receiving 0 p.p.m. lead acetate (5.5×10^6 parasites/ml of blood) (Figure 5). All of the above results were significantly different.

A previous study by Grogl and Kuhn (1985) compared the course of T. cruzi infection in relatively resistant C57Bl/6 mice to that in C3H(He) mice, which are highly susceptible to infection. Both strains of mice were inoculated with 1×10^3 BFT of T. cruzi. It was reported that C3H(He) mice developed higher parasitemias than C57Bl/6 mice and that parasite numbers increased faster in C3H(He) mice. C3H(He) mice died with high parasitemias (1.6×10^6 parasites/ml of blood) by day 24 or 25 post infection, whereas C57Bl/6 mice survived from 46-180 days post infection. C57Bl/6 mice had peak parasitemias ranging from 5.0×10^5 to 9.0×10^5 parasites/ml of blood. In the present study, mice in the control group (0 p.p.m. lead acetate) had a mean peak parasitemia of 6.49×10^6 parasites/ml of blood. Observed numbers of circulating BFTs in the present study may have been higher than those described by Grogl and Kuhn (1985) because a tenfold greater parasite density was used for inoculation.

The high parasitemias observed in the present study suggest that lead-treated C57Bl/6 mice are more susceptible to T. cruzi infection. However, despite the pronounced effect of high lead acetate concentrations on parasitemias, no increase in mortality was observed. The question of why the animals survived up to 275 days post infection with chronic exposure to high concentrations of lead acetate and high parasitemias still remains unanswered. The effects of lead exposure on parasites are unknown but lead may play an important role in altering the pathogenicity of the parasite.

Immediately following the peak parasitemias in all three replicate experiments, there was a sharp decrease observed in parasite counts. Antibody-dependent complement-mediated lysis of trypomastigotes may be responsible for this rapid decline. Despite severe immunosuppression observed during the acute stage of Chagas' disease, it is well known that the parasite-specific antibodies produced during the course of infection have a protective effect and demonstrate lytic activity against the parasite (Leguizamon *et al.*, 1991).

To examine the effects of lead exposure on the humoral immune response to T. cruzi, parasite-specific antibodies were measured during the course of infection. In contrast to previous studies (Koller and Kovacic, 1974; Blakley *et al.*, 1980) which suggested that lead salts suppress antibody production, in this study all three replicate experiments showed no obvious pattern between high lead acetate exposure and lowered parasite-specific antibody production (Figure 6).

The results from replicate experiment I suggest highest levels of anti-T. cruzi

antibodies are being produced in mice receiving 100 and 1000 p.p.m. lead acetate just prior to peak parasitemias (Figures 6 and 5, respectively). In replicates II and III, highest levels of anti-T. cruzi antibodies in mice receiving 100 and 1000 p.p.m. lead acetate were observed between 20 to 27 days after the peak of circulating BFTs (Figure 6 and 5, respectively). Although, extremely high numbers of parasites were observed in the present study there was no significant correlation between parasitemias and antibody production. This result is in contrast to a previous study by Bennett (1995) which demonstrated that parasitemias had a significantly positive correlation with levels of anti-T. cruzi antibodies in C3HeB/FeJ mice.

The results from the present study suggest lead acetate exposure time (21 days) prior to T. cruzi infection may not be long enough to cause decreased antibody production in this less susceptible mouse strain subsequent to infection. In previous studies in which decreased B-cell and antibody production was seen, experimental animals were exposed to lead salts for 21 (Blakley *et al.*, 1980), 49 (Faith *et al.*, 1979), 56 (Koller and Kovacic, 1974), or 70 days (Koller, 1973; Koller and Brauner, 1977) prior to antigen exposure. In the present study, anti-T. cruzi antibody levels were not measured late in infection (after 51 days post infection) and after chronic lead acetate exposure. It is possible that a decrease in parasite-specific antibody production might have been observed in later stages of the infection.

In a previous study by Grogl and Kuhn (1985), parasite-specific antibody production was measured in C3H(He) mice and C57Bl/6 mice during T. cruzi infection. This study showed C57Bl/6 mice produced two times more anti-T. cruzi

antibodies than C3H(He) mice. Immunoglobulin IgG, which is the primary antibody produced during the course of T. cruzi infection, peaked on days 20 and 48 for C3H(He) and C57Bl/6 mice, respectively. Immunoglobulin IgM, peaked on day 20 and days 25 and 40 for C3H(He) and C57Bl/6 mice, respectively. The authors concluded that the high titer of anti-T. cruzi antibody in C57Bl/6 mice was correlated with their increased resistance to T. cruzi infection (Grogl and Kuhn, 1985). Parasite-specific antibody production was highest on days 25, 40, and 48 post infection in C57Bl/6 mice (Grogl and Kuhn, 1985); therefore, supporting the results from the present study which show an increase in antibody production on days 46 and 51 post infection in replicates II and III, respectively.

Daily water and food consumption from each treatment group was measured periodically throughout the study. After conducting a regression analysis on the data, all slopes were shown to be negative, demonstrating a significant decrease in both water and food consumption over time. Replicate by treatment combinations show most lead-treatment groups had comparable decreases in water and food consumption, therefore, suggesting mice per cage received similar amounts of water and food and appropriate lead acetate concentrations. The observed decrease in water and food consumption for all lead-treated groups of mice was likely due to the progressive decrease in activity which was observed in mice infected with T. cruzi. When data from all treatments were pooled, 100 p.p.m. lead-treated mice drank significantly more water than either Groups 2 (10 p.p.m.) or 4 (1000 p.p.m.) in replicate experiment I. No significance in among lead-treated groups was observed in replicates II and III.

Results from Blakley and Archer (1981) and Blakley *et al.* (1980) also demonstrated that water consumption in lead-treated mice measured over 3 weeks was not negatively impacted by lead exposure.

Individual mouse weights were measured during the course of the study. In replicate I, 100 p.p.m. lead acetate-treated mice weighed significantly more than mice treated with 1000 p.p.m. lead acetate concentrations (Figure 1); however, no statistical differences were observed among lead-treated groups in replicates II or III. Mice in replicate I receiving 100 p.p.m. may have weighed more because they drank significantly more water than mice receiving 1000 p.p.m. (Figure 2). A noticeable drop in the weight of mice receiving 1000 p.p.m. occurred between days 50 and 181 in replicate II. Such a large decline in weight could be due to a temporary water loss in the mice. However, the long interval between weight measurements makes it difficult to interpret this result. In all three replicate experiments mice reached a plateau in weight between days 50 and 60, suggesting individual growth rates were similar among groups. These results are supported by the studies of Blakley *et al.* (1980) and Blakley and Archer (1981) who showed there was no significant effect of lead treatment on weight gain of Swiss cross mice or BDF_1 mice, respectively. Similarly Sin and Woo (1992) reported that mean body weights of lead-treated Swiss albino mice were not significantly different when compared to the control group. However, one study by Koller (1973) showed New Zealand White rabbits exposed to 2500 p.p.m. lead acetate concentrations weighed significantly less than non-lead-treated control rabbits.

Mice were observed for signs of Chagas' disease and lead toxicity. Late in infection 100 and 1000 p.p.m. lead acetate-treated mice became lethargic and characteristic "humped backs" were observed along with weakened hind legs. Toxicological signs observed were predominantly in mice treated with 100 and 1000 p.p.m. lead acetate which included hair loss and what was thought to be neurological damage. The only observed mortality in the study was one mouse (in replicate I) receiving 1000 p.p.m. lead acetate which died on day 63 post infection with low body weight and obvious signs of Chagas' disease.

The results from the present study support previous studies which have suggested that exposure to lead increases susceptibility to infectious agents (Hemphill *et al.*, 1971; Koller, 1973; Koller and Kovacic, 1974; Lawrence, 1981; and Sin and Woo, 1992). C57Bl/6 mice exposed to 100 and 1000 p.p.m. lead acetate concentrations had mean peak parasitemias which were significantly higher when compared to control mice and respectively, nineteen times and fifty-two times higher than peak parasitemias of non-lead-treated, T. cruzi infected, C57Bl/6 mice observed by Grogl and Kuhn (1985). Tissue levels of parasites and tissue destruction also would be expected to be greater in mice with higher parasitemias, however, histological studies will be necessary to confirm this prediction.

It will be important for future studies to determine the effects of lead acetate exposure on $CD4^+$ T helper and $CD8^+$ T cytotoxic activity during T. cruzi infection. Faith *et al.* (1979) demonstrated exposure to lead salts had a negative impact on cell-mediated immunity by decreasing T-cell proliferation. Lead has also been shown to

affect $CD4^+$ T-cell function (McCabe and Lawrence, 1990) and inhibit T_h 1 subpopulation function (Kowolenko *et al.*, 1992).

As a follow-up to the present study, the laboratory will evaluate the effects of lead acetate exposure in C3H(He) mice, which are highly susceptible to challenge with T. cruzi. This project will evaluate the effect of lead exposure on parasitemia and mortality, evaluate the effects of lead exposure and T. cruzi on cardiac and skeletal muscle, liver, and kidney tissues, and evaluate water and weight changes of the lead-treated mice when infected with T. cruzi.

Since 1970 many natural and synthetic substances have been shown to alter the immune response and cause increased susceptibility to disease. With the rapid progression of technology, it is important to be cognizant of the continuous work of pharmaceutical companies (producing "newer and better" drugs) and chemical companies (producing pesticides, synthetic fibers, etc.) as we approach the 21st century. Due to such environmental health concerns, the past 20 years have been important in evaluating environmental contaminants and toxicants which may have a negative impact on the immune response therefore increasing susceptibility to infectious agents. However, little research has been done to evaluate the effects of environmental toxicants during parasitic infection. The topic of parasitic disease receives little attention in the U. S., however, an estimated one billion of the world's population is currently suffering from parasitic infection (Goodman, 1994). Furthermore, infectious and parasitic diseases account for more than half the total burden of morbidity and mortality in Sub-Saharan Africa, India, and much of Asia (Evans and Jamison, 1994).

Therefore, in light of the results of the present study, future studies should be designed to further evaluate the effect of environmental toxicants, such as lead, during parasitic infection.

LITERATURE CITED

Bennett, B. J. 1995. The effect of dietary supplemented vitamin E and selenium on experimental Chagas' disease. Master's Thesis. Western Kentucky University, Bowling Green, KY. p. 28.

Blakley, R. R., C. S. Sisodia, and T. K. Mukkur. 1980. The effect of methylmercury, tetraethyl lead and sodium arsenite on the humoral immune response in mice. Toxicology and Applied Pharmacology **52:** 245-254.

Blakley, R. R., and D. L. Archer. 1981. The effect of lead acetate on the immune response in mice. Toxicology and Applied Pharmacology **61:** 18-26.

Bogitsh, B. J., and T. C. Cheng. 1990. Blood and Tissue Protozoa I: Hemoflagellates. In Human Parasitology, Saunders College Publishing, New York, New York, 105-109.

Calebrese, K. S., P. G. Bauer, P. H. Lagrange, and S. Goncalves da Costa. 1991. Trypanosoma cruzi infection in immunosuppressed mice. Immunology Letters **31:** 91-96.

Caren, L. D. 1981. Environmental pollutants: Effects on the immune system and resistance to infectious disease. Bioscience **31(8):** 582-586.

Evans, D. B. and D. T. Jamison. 1994. Economics and the argument for parasitic disease control. Science **264:** 1866-1867.

Exon, J. 1990. Concepts and definition of immunotoxicology. In Immunotoxicology of Metals and Immunotoxicology, A. Dayan (ed). Plenum, New York, New York, p. 29.

Faith, R. E., M. I. Luster, and C. A. Kimmel. 1979. Effect of chronic developmental lead exposure on cell-mediated immune functions. Clinical and Experimental Immunology **35:** 413-420.

Faith, R. E., M. I. Luster, and J. G. Vos. 1980. Effects on immunocompetence by chemicals of environmental concerns. In Reviews in Biochemical Toxicology, vol. 2, E. Hodgson (ed). Elsevier North Holland, New York, New York, p. 173-211.

Goodman, B. 1994. Models aid understanding, help control parasites. Science **264:** 1862-1863.

Goyer, R. 1991. Toxic Effects of Metals. In Casarett and Doull's Toxicology: The Basic Science of Poisons, 4th Ed. M. O. Amdur (ed). McGraw-Hill, New York, New York, p.623-680.

Grogl, M. and R. E. Kuhn. 1985. Identification of antigens of Trypanosoma cruzi which induce antibodies during experimental Chagas' disease. Journal of Parasitology **71 (2):** 183-191.

Hemphill, F. E., M. L. Kaeberle, and W. B. Buck. 1971. Lead suppression of mouse resistance to Salmonella typhimurium. Science **172:** 1031-1032.

Hilbertz, U., U. Kramer, N. DeRutter, and B. Baginski. 1986. Effects of cadmium and lead on oxidative metabolism and phagocytosis by mouse peritoneal

macrophages. Toxicology **39:** 47-57.

Koller, L. D. 1973. Immunosuppression produced by lead, cadmium, and mercury. American Journal of Veterinary Research **34:** 1457-1458.

Koller, L. D. 1979. Effects of environmental contaminants on the immune system. Advances in Veterinary Science and Comparative Medicine **23:** 267-295.

Koller, L. D. 1980. Review: Immunotoxicology of heavy metals. International Journal of Immunopharmacology **2:** 269-279.

Koller, L. D. and J. A. Brauner. 1977. Decreased B-lymphocyte response after exposure to lead and cadmium. Toxicology and Applied Pharmacology **41:** 621-624.

Koller, L. D. and S. Kovacic. 1974. Decreased antibody formation in mice exposed to lead. Nature **250:** 148-149.

Koller, L. D., J. G. Roan, and N. I. Kerkvliet. 1979. Mitogen stimulation of lymphocytes in CBA mice exposed to lead and cadmium. Environmental Research **19:** 177-188.

Kowolenko, M., M. J. McCabe Jr., and D. A. Lawrence. 1992. Metal-induced alterations of immunity. In Clinical Immunotoxicology, D. S. Newcombe, N. R. Rose, and J. Bloom (eds.). Raven Press, New York, New York, p. 401-419.

Kuby, J. 1994. Immunology, 2nd ed. W. H. Freeman and Company, New York, New York, p. 4, 11, and 607.

Kuhn, R. E. 1989. Immunology and immunoregulation of parasitic hematozoan infection. American Zoologist **29:** 427-440.

Lawrence, D. A. 1981. Heavy metal modulation of lymphocyte activities. Toxicology and Applied Pharmacology **57:** 439-451.

Leguizamon, M. S., O. E. Campetella, M. B. Reyes, C. F. Ibanez, M. A. Basombrio, J. Rincon, A. Orn, and A. C. C. Frasch. 1991. Bloodstream Trypanosoma cruzi parasites from mice simultaneously express antigens that are markers of acute and chronic human Chagas' disease. Parasitology **102:** 379-385.

Lima, M., H. L. Lenzi, and C. R. Gattass. 1995. Negative tissue parasitism in mice injected with a noninfective clone of Trypanosoma cruzi. Parasitology Research **85:** 6-12.

Loblay, R. and S. Neill. 1992. Immunotoxicology. In Encyclopedia of Immunology, vol. 2. Academic Press, London U. K., p. 860.

Luster, M. I., J. H. Dean, and J. A. Moore. 1984. Evaluation of immune functions in toxicology. In Principles and Methods in Toxicology, A. W. Hayes (ed.). Raven Press, New York, New York, p. 561.

Markell, E. K., M. Voge, and D. T. John. 1986. Other blood- and tissue-dwelling protozoa. In Medical Parasitology, 6th ed. W. B. Saunders, Philadelphia, Pennsylvania, p. 112-121.

McCabe, M. J. and D. A. Lawrence. 1990. The heavy metal lead exhibits B-cell stimulatory factor activity by enhancing B-cell Ia expression and differentiation. Journal of Immunology **145:** 671-677.

Miles, M. A. 1983. The epidemiology of South American Trypanosomiasis-Biochemical and immunological approaches and their relevance to control.

Transactions of the Royal Society of Tropical Medicine and Hygiene **77 (1):** 5-23.

Minchin, E. A. 1910. A new trypanosome parasitic in human beings. Nature **84:** 142-144.

Newcombe, D. 1992. Immunotoxicology: A new challenge. In Clinical Immunotoxicology, D. Newcombe *et al.*, (eds.). Raven Press, New York, New York, p. 1.

Rowland, E. C., M. G. Lozykowski, and T. S. McCormick. 1992. Differential cardiac histopathology in inbred mouse strains chronically infected with Trypanosoma cruzi. Journal of Parasitology **78 (6):** 1059-1066.

Seyle, H., B. Tuchweber, and L. Betock. 1966. Effect of lead acetate on the susceptibility of rats to bacterial endotoxins. Journal of Bacteriology **91:** 884-890.

Sin, Y. M. and P. T. Woo. 1992. Inflammatory response and resistance in lead-treated mice. Bulletin of Environmental Contamination and Toxicology **48:** 502-508.

Sun, J., and R. L. Tarleton. 1993. Predominance of $CD8^+$ T lymphocytes in the inflammatory lesions of mice with acute Trypanosoma cruzi infection. The American Journal of Tropical Medicine and Hygiene **48 (2):** 161-168.

Tarleton, R. L. 1990. Depletion of $CD8^+$ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with Trypanosoma cruzi. Journal of Immunology **144 (2):** 714-724.

Vos, J. 1977. Immune suppression as related to toxicology. CRC Critical Reviews in Toxicology, p. 67-101.

Wendel, S. and A. L. Gonzaga. 1993. Chagas' disease and blood transfusions: A new world problem? V. Sanguinis (ed.), **64:** 1-12.

White, K. L. Jr. 1992. Specific immune function assays. In Principles and Practices of Immunotoxicology, K. Miller *et al.*, (eds.). Blackwell Scientific Publications, London, U. K. p. 304-323.